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Note

Evaluation of a Fluorescence Hybridisation Assay Using Peptide Nucleic Acid Probes for Identification and Differentiation of Tuberculous and Non-Tuberculous Mycobacteria in Liquid Cultures

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Abstract The performance was evaluated of a fluorescence in situ hybridisation assay using peptide nucleic acid probes (Dako Probe MTB Culture Confirmation Test; Dako, Denmark) for identification of *Mycobacterium tuberculosis* complex (MTC) organisms and differentiation between tuberculous and non-tuberculous mycobacteria (NTM) in material taken directly from Bactec 12B (Becton Dickinson, USA) and MB/BacT (Organon Teknika, USA) bottles. The test was applied to 129 smear-positive (Ziehl-Neelsen stain) clinical specimens, 48 previously identified clinical strains of mycobacteria (12 MTC and 36 NTM), and 51 reference strains (7 MTC and 44 NTM) which were all previously inoculated into Bactec 12B and MB/BacT bottles. The sensitivity and specificity of the assay for MTC-positive cultures was 87.6% and 100%, respectively, for Bactec 12B, and 100%, respectively, for MB/BacT. The sensitivity and specificity of the assay for NTM-positive cultures was 100% for both media.

Introduction

Standard methods for isolation and identification of mycobacteria require up to 6–8 weeks for growth and several more weeks for identification [1]. The development of DNA probes has contributed considerably to the rapid identification of *Mycobacterium* species in cultures of clinical specimens. The AccuProbe (GenProbe, USA), which employs acridinium ester as the probe label, has been extensively evaluated in the identification of organisms isolated on solid media [2, 3]. In other studies the applicability of this assay in the identification of organisms obtained directly from liquid culture media has been investigated [4–6].

Peptide nucleic acids (PNA) are novel DNA-like compounds, in which nucleotide bases are attached via methylene-carbonyl links to a peptide backbone consisting of N-2-aminoethylglycine units [7]. Several features of PNA, such as superior hybridisation affinity to RNA and DNA, high stability and convenient solid-phase synthesis, make them promising candidates for use in molecular biology, nucleic acid diagnostics and therapeutics [8, 9].

The Dako Probe (Dako Probe MTB Culture Confirmation Test; Dako, Denmark) is a new molecular method based on fluorescein-labelled PNA probes for the separate detection of *Mycobacterium tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM) by fluorescence in situ hybridisation (TB PNA FISH). In this study, we evaluated the performance of the Dako Probe in the identification and differentiation of MTC and NTM in material taken directly from the radiometric Bactec 460 TB and the non-radiometric MB/BacT broth culture systems.

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Materials and Methods

In the period from June 1998 to January 1999, 129 smear-positive (Ziehl-Neelsen stain) clinical specimens from 112 patients were collected for the study. The specimens included expectorated sputum ($n=89$), bronchial aspirate ($n=16$), bronchoalveolar lavage ($n=7$), faeces ($n=6$), urine ($n=2$), tissue biopsy material ($n=2$), lymph node exudate ($n=5$) and bone marrow aspirate

($n = 2$). The specimens were digested and decontaminated with sodium dodecyl (lauryl) sulfate (SDS)-NaOH using the standard method of Tacquet and Tison, as outlined elsewhere [10]. The resulting sediment was washed with 30 ml of distilled water and centrifuged (3300 × g for 20 min), and the supernatant was removed. The cell pellet of all pretreated specimens was resuspended in 1.5 ml of distilled water.

A total of 48 mycobacterial strains from clinical specimens previously identified in our laboratory were included in the study. These strains comprised two *Mycobacterium avium*, four *Mycobacterium kansasii*, two *Mycobacterium simiae*, four *Mycobacterium scrofulaceum*, four *Mycobacterium intracellulare*, four *Mycobacterium gordonae*, four *Mycobacterium gastri*, one *Mycobacterium szulgai*, three *Mycobacterium xenopi*, two *Mycobacterium flavescens*, two *Mycobacterium peregrinum*, two *Mycobacterium fortuitum*, two *Mycobacterium smegmatis*, two *Mycobacterium africanum* and ten *Mycobacterium tuberculosis* strains.

A total of 51 mycobacterial isolates derived from 17 reference strains were also included in the study. These mycobacterial isolates comprised four *Mycobacterium intracellulare* (ATCC 13950), two *Mycobacterium fortuitum* (ATCC 6841), three *Mycobacterium simiae* (ATCC 25275), four *Mycobacterium scrofulaceum* (ATCC 19981), two *Mycobacterium xenopi* (NCTC 10042), four *Mycobacterium kansasii* (ATCC 12478), four *Mycobacterium gordonae* (ATCC 14470), two *Mycobacterium smegmatis* (ATCC 19420), four *Mycobacterium gastri* (ATCC 15754), four *Mycobacterium avium* (ATCC 25291), two *Mycobacterium terrae* (ATCC 15755), two *Mycobacterium peregrinum* (ATCC 14467), two *Mycobacterium flavescens* (ATCC 14474), three *Mycobacterium szulgai* (NCTC 10831), two *Mycobacterium vaccae* (ATCC 15483), two *Mycobacterium bovis* (BCG) and five *Mycobacterium tuberculosis* (ATCC 27294) strains.

For processing of all clinical strains, 500 µl of the processed sediment was inoculated onto Lowenstein-Jensen and Coletsos solid slants, and used for acid-fast staining. In addition, 500 µl of the sediment was inoculated into the liquid media Bactec 12B and MB/BacT.

For processing of clinical and reference strains, a few representative colonies of each strain were removed with a loop from the Lowenstein-Jensen slant and suspended in 5 ml of distilled water. The suspension was thoroughly homogenised with glass beads (Vortex) and adjusted by dilution with distilled water to an optical density equivalent to a McFarland No. 1 standard. Tenfold dilutions (approximately 10^5 cells/ml) were performed in 5 ml sterile glass test-tubes. Finally, 100 µl of this suspension was inoculated into each Bactec 12B and MB/BacT bottle. A Bactec growth index of 999 was considered to indicate positivity. Smears for Ziehl-Neelsen staining and culture on solid media were prepared from all positive liquid culture bottles.

The target of the PNA probes in the Dako Probe is mycobacterial 16S rRNA. The kit includes two PNA probes: an MTB probe targeting species of the *Mycobacterium tuberculosis* complex and an NTM probe targeting most clinically relevant NTM including *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium paratuberculosis*, *Mycobacterium scrofulaceum*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium malmoense*, *Mycobacterium gastri*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium simiae*, *Mycobacterium szulgai*, and *Mycobacterium haemophilum*.

As soon as mycobacterial growth was detected in each broth culture system, an aliquot was obtained for acid-fast staining by the Ziehl-Neelsen method. Once isolates were confirmed to be acid-fast, 1 ml of broth culture was removed and transferred to an Eppendorf tube. The tube was centrifuged for 5 min at 5000 × g, and the supernatant was removed. The pellet was resuspended in 200 µl of PBS buffer. Approximately 25 µl of this mycobacterial suspension was smeared onto both the left and right well of a three-well microscope slide. One slide was used for the standard hybridisation and a second slide was used to repeat the assay if both smears were either negative or positive (quality control slide). The smears were allowed to air-dry and fixed by immersion of the slide in absolute methanol for 1 min. Just prior to the hybridisation procedure, the smears were immersed in 80% ethanol for 15 min and then air-dried for another 10 min.

A fluorescein-labelled MTB-specific PNA probe and a fluorescein-labelled NTM-specific PNA probe were added to the two mycobacterial smears and incubated at 55°C for 90 min for hybridisation to rRNA in the mycobacterial cells. The hybridisation step was followed by a post-hybridisation wash using a stringent wash solution at 55°C for 30 min. Slides were finally immersed in distilled water at room temperature for 30 sec. Mycobacterial smears were subsequently mounted with mounting medium and incubated at 55°C for 30 min. Finally, the smears were examined by fluorescence microscopy with a Nikon Eclipse E-400 fluorescence microscope (Nikon Corporation, Japan) equipped with a 100 W mercury light source, using a FITC/Texas Red double filter (Chroma Technology, USA) and a 100× Plan-Fluor oil lens (Nikon Corporation) with a 1.3 numerical aperture. Mycobacteria were detected by fluorescence emission. In the case of positive results, both the number of stained mycobacteria [34] and the fluorescence intensity were scored, the scores for the latter being as follows: +/−: very weak; +: weak; ++: moderate; +++: strong; ++++: very strong.

For each sample, one of the two smears tested with the MTB probe and the NTM probe had to be positive, whereas the other smear had to be negative. This ensured that smears contained mycobacterial cells and that hybridisation conditions were satisfactory. If positive hybridisation results were obtained with both the MTB and NTM probes, the test result was considered inconclusive and the assay was repeated. Similarly, if a negative result was obtained with both the MTB and NTM probes, the test result was considered inconclusive and Ziehl-Neelsen staining was performed on both smears to determine the presence of any acid-fast bacilli on the slide.

Routine biochemical methods [1], gas-liquid chromatography [11], thin-layer chromatography [11], and the AccuProbe Culture Confirmation Test (Gen-Probe, USA) [2] were employed to identify clinical isolates.

Subcultures from Bactec 12B- and MB/BacT-positive bottles were examined for purity and for agreement with the results of the Dako Probe. Discrepant results were analysed by repeating the Dako Probe test directly with colonies from each subculture isolate.

Results and Discussion

Of the 129 clinical specimens examined, 97 were culture-positive for *Mycobacterium tuberculosis* and 32 for non-tuberculous mycobacteria (NTM). The 32 NTM isolates comprised *Mycobacterium avium* (15 specimens), *Mycobacterium kansasii* (10 specimens), *Mycobacterium simiae* (2 specimens), *Mycobacterium scrofulaceum* (1 specimen), *Mycobacterium intracellulare* (1 specimen), *Mycobacterium gordonae* (1 specimen) and *Mycobacterium xenopi* (2 specimens).

The Dako Probe detected 85 (87.6%) of the isolates in the 97 Bactec 12B bottles positive for MTC. None of these isolates hybridised with the NTM probe. Negative

hybridisation results were obtained for both MTB and NTM probes when the Dako Probe was applied to 12 positive Bactec 12B cultures containing MTC isolates. Similarly, negative hybridisation results were obtained when the Dako Probe was applied to the 12 corresponding quality control slides. A possible explanation for these false-negative results could be the loss of mycobacteria from the slide during the hybridisation procedure. The few acid-fast bacilli observed on these processed slides when Ziehl-Neelsen staining was performed confirms this conjecture. Positive hybridisation results were obtained in all 12 negative specimens when a new aliquot of each Bactec 12B medium was tested after 2 additional days of incubation. This indicates that for optimal hybridisation results a large number of mycobacteria fixed in the smears is required to counteract the loss of bacteria during the hybridisation technique.

The overall sensitivity and specificity of the Dako Probe for positive Bactec 12B cultures containing MTC isolates was 87.6% and 100%, respectively (Table 1). Similar results were reported for the AccuProbe using material taken directly from Bactec 12B broth cultures [4]. However, the results were better than those reported in several studies in which the AccuProbe was directly applied to material from liquid mycobacteria cultures [6, 12, 13]. Of the 85 positive hybridisation results obtained for positive Bactec 12B bottles containing MTC isolates, 22 (22.6%) showed weak or moderate fluorescence and 63 (64.9%) strong fluorescence.

All MTC isolates in the 97 positive MB/BacT cultures containing MTC clinical isolates were detected using the Dako Probe. None of these isolates hybridised with the NTM probe. The overall sensitivity and specificity of the Dako Probe for positive MB/BacT cultures containing MTC clinical isolates was 100%, respectively (Table 1). Six (6.1%) of these 97 positive isolates showed weak or moderate fluorescence and 91 (93.8%) strong fluorescence.

The Dako Probe detected 30 (93.7%) of the isolates in the liquid medium of the 32 positive Bactec 12B and MB/BacT bottles containing NTM isolates. None of these isolates hybridised with the MTB probe. Two of the 32 bottles contained mycobacteria not identifiable with the NTM probe (*Mycobacterium xenopi*); thus, the isolates were not detected by the Dako Probe. Results for these two isolates were not considered false-negative and were not included in sensitivity calculation. The overall sensitivity and specificity of the test for positive Bactec 12B and MB/BacT bottles containing NTM isolates was 100%, respectively (Table 1). All 30 positive hybridisation results for NTM clinical isolates showed strong or very strong fluorescence in both liquid media.

A total of 51 isolates derived from reference strains of several mycobacterial species were tested, including seven MTC (5 *Mycobacterium tuberculosis* ATCC 27294 and 2 *Mycobacterium bovis*-BCG) and 44 NTM. The Dako Probe detected 30 (68.1%) of the NTM isolates in liquid medium from the 44 positive Bactec 12B and MB/BacT bottles containing NTM reference strains. None of these isolates hybridised with the MTB probe. These 44 NTM isolates comprised 14 mycobacterial species (2 *Mycobacterium fortuitum*, 2 *Mycobacterium xenopi*, 2 *Mycobacterium smegmatis*, 2 *Mycobacterium terrae*, 2 *Mycobacterium peregrinum*, 2 *Mycobacterium flavescens*, and 2 *Mycobacterium vaccae*) not detected by the Dako Probe. Since the NTM probe is not designed to hybridise with these mycobacteria, the results were not considered false-negative. Five Bactec 12B and MB/BacT bottles were positive for *Mycobac-*

Table 1 Results of direct testing of positive Bactec 12B and MB/BacT bottles with the Dako Probe

Source	Organism	No. of cultures	Positive Dako Probe test results				Sensitivity (%)		Specificity (%)	
			No. (%) positive with MTB probe		No. (%) positive with NTM probe					
			Bactec 12B	MB/BacT	Bactec 12B	MB/BacT	Bactec 12B	MB/BacT	Bactec 12B	MB/BacT
Clinical specimens	MTC	97	85 (87.6)	97 (100)	0 (0)	0 (0)	87.6	100	100	100
	NTM	32[a,f]	0 (0)	0 (0)	30 (93.7)	30 (93.7)	100	100	100	100
Reference strains	MTC[b]	7	7 (100)	7 (100)	0 (0)	0 (0)	100	100	100	100
	NTM	44[c,f]	0 (0)	0 (0)	30 (68.1)	30 (68.1)	100	100	100	100
Mycobacterial strains	MTC[d]	12	12 (100)	12 (100)	0 (0)	0 (0)	100	100	100	100
	NTM	36[e,f]	0 (0)	0 (0)	25 (69.4)	25 (69.4)	100	100	100	100

[a] Includes two *M. xenopi* isolates
[b] 5 *M. tuberculosis* and 2 *M. bovis* strains
[c] 2 *M. xenopi*, 2 *M. vaccae*, 2 *M. flavescens*, 2 *M. smegmatis*, 2 *M. peregrinum*, 2 *M. fortuitum*, and 2 *M. terrae*
[d] 10 *M. tuberculosis* and 2 *M. africanum* strains
[e] 3 *M. xenopi*, 2 *M. flavescens*, 2 *M. peregrinum*, 2 *M. smegmatis*, and 2 *M. fortuitum*
[f] Because the NTM probe is not designed to hybridise with these NTM species, these results were not included in sensitivity calculation
MTC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria

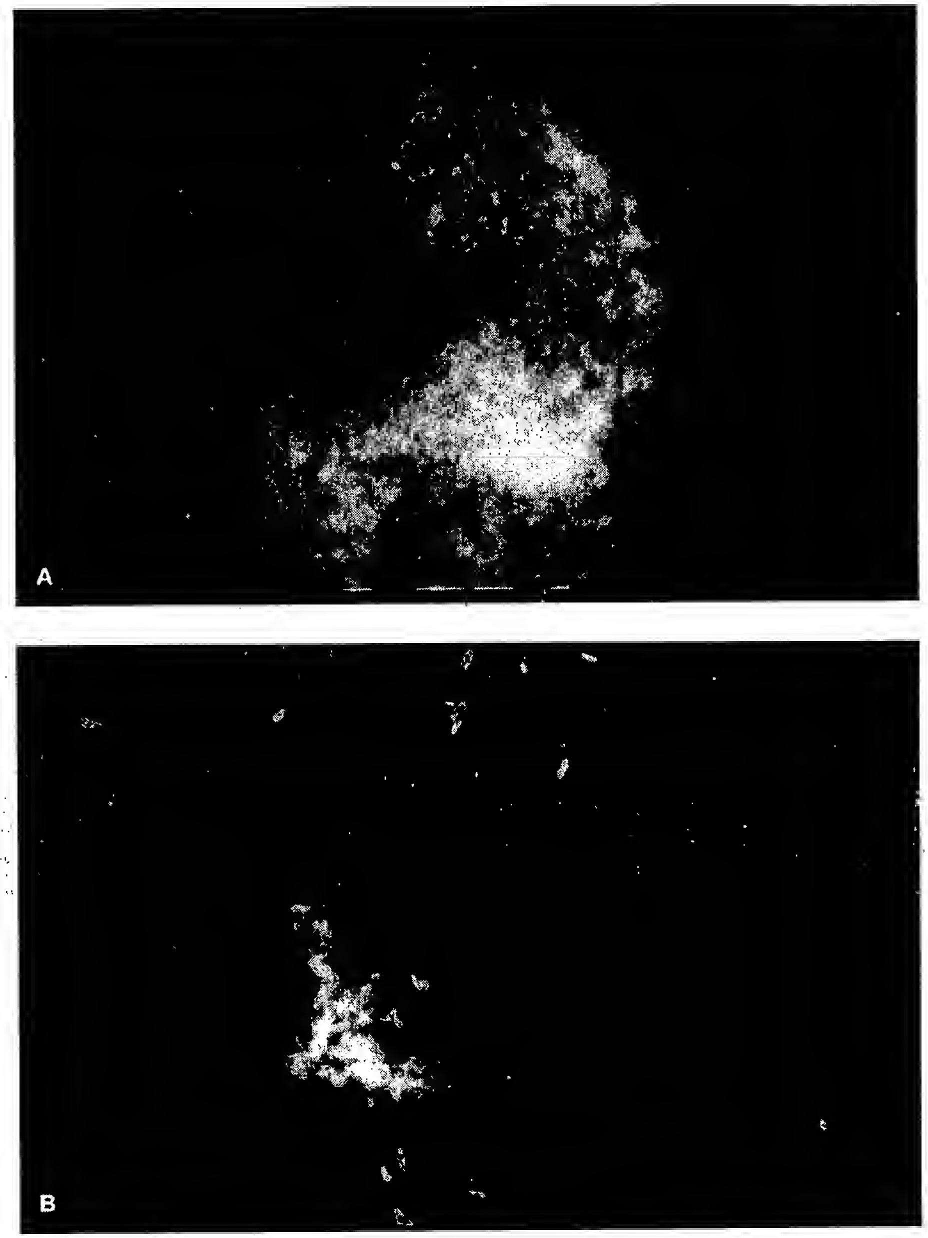
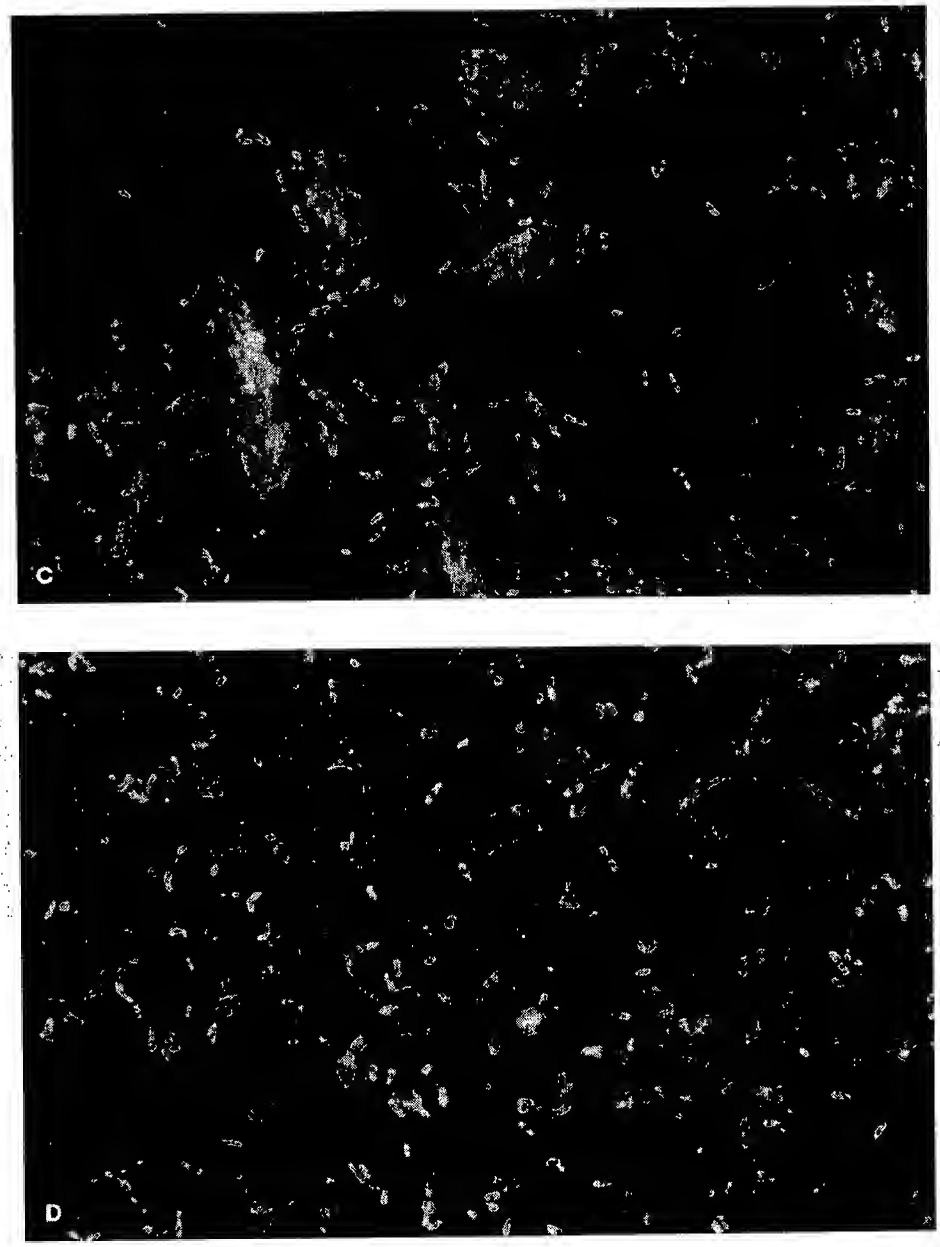


Figure 1 Microscopic morphology of different species of *Mycobacterium* after applying the Dako Probe (magnification × 1000). **A** *Mycobacterium tuberculosis*. **B** *Mycobacterium bovis*. **C** *Mycobacterium kansasii*. **D** *Mycobacterium gordonae*

terium tuberculosis (ATCC 9360) and two for *Mycobacterium bovis* (BCG), all these mycobacteria being detected by the Dako Probe. The overall sensitivity and specificity of the Dako Probe for both NTM and MTC reference isolates was 100%, respectively, in both liquid media (Table 1).

A total of 48 clinical mycobacterial strains previously identified in our laboratory were also tested, including 36 NTM and 12 MTC. The Dako Probe detected 25 (69%) of the NTM isolates in the 36 Bactec 12B and MB/BacT bottles positive for these clinical NTM strains. None of these isolates hybridised with the MTB probe. The 11 isolates not detected by the Dako Probe included 2 *Mycobacterium fortuitum*, 3 *Mycobacterium xenopi*, 2 *Mycobacterium smegmatis*, 2 *Mycobacterium peregrinum* and 2 *Mycobacterium flavescens* isolates. Since the NTM probe is not designed to hybridise with these mycobacteria, the results were not considered false-negative. There were 12 positive Bactec 12B and MB/BacT cultures containing clinical MTC strains (10 *Mycobacterium tuberculosis* and 2 *Mycobacterium africanum*). The Dako Probe was able to detect all of these isolates. None of them hybridised with the NTM probe. The overall sensitivity and specificity of the Dako Probe for both NTM and MTC was 100%, respectively, in both liquid culture media (Table 1).

Figure 1 (cont.)



In the present study, we obtained a sensitivity of 87.6% and specificity of 100% using the Dako Probe to detect MTC isolates in positive Bactec 12B cultures of clinical specimens. However, we obtained 100% sensitivity and specificity when the probe was used to detect NTM isolates from different sources (clinical specimens, clinical strains and reference strains) in Bactec 12B medium. Excellent (100%) sensitivity and specificity were achieved using the Dako Probe to detect MTC and NTM isolates from different sources (clinical specimens, clinical strains and reference strains) in MB/BacT medium. Thus, we obtained better results using the Dako Probe to detect MTC in MB/BacT than in Bactec 12B medium. The fact that a positive MB/BacT bottle contains a larger number of mycobacteria than a positive Bactec 12B culture may explain the difference in sensitivity between the two media.

A preliminary version of the Dako Probe has been recently evaluated in 30 Bactec 12B cultures positive for acid-fast bacilli [14]. In that study, the MTB probe showed a sensitivity and specificity of 84% and 100%, respectively, whereas the NTM probe showed a sensitivity and specificity of 91% and 100%, respectively. These results are similar to those obtained in our study when the test was performed on Bactec 12B cultures.

In general, the results obtained in our study with the Dako Probe were better than those reported for the AccuProbe for the direct testing of liquid cultures containing MTC isolates [6, 12, 13]. In addition, the Dako Probe allowed differentiation between MTC isolates and all those NTM isolates of species identifiable with the NTM probe. Unlike DNA probes, the Dako Probe allows direct detection of MTC and most clinically relevant NTM in a single test. It also facilitates the use of mycobacterial morphology as an additional identification tool through microscopic analysis (Figure 1). The test can easily be adapted for incorporation in microscopy techniques used in most clinical microbiology laboratories; the only special requirement being that an FITC/Texas Red double filter be fitted onto the fluoresecence microscope. When a standard FITC filter is used, the autofluorescence of the smear itself makes it practically impossible to distinguish positive from negative results. Better fluorescence results were obtained when a 100 W rather than a 50 W lamp microscope was used to observe the fluorescence intensity of the slides.

The Dako Probe also has several disadvantages. One disadvantage is the fact that a large number of mycobacteria is required to obtain optimal sensitivity results. This only affected the test when it was performed on positive Bactec 12B cultures containing *Mycobacterium tuberculosis* isolates. A further disadvantage is the fact that the Dako Probe can only be used in positive Bactec 12B cultures when the growth index reaches 999, which could mean a delay in diagnosing tuberculosis, whereas the AccuProbe can be applied at an earlier stage of mycobacterial growth. Finally, although the Dako Probe has been shown to be an excellent test for identification and differentiation of MTC and NTM in material taken directly from primary liquid cultures, it would be desirable to extend the detection capacity of the NTM probe to include more non-tuberculous mycobacteria, especially *Mycobacterium xenopi*.

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